

Poly(ADP-ribosylation) of DNA Topoisomerase I from Calf Thymus*

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We demonstrate that the activity of the major DNA topoisomerase I from calf thymus is severely inhibited after modification by purified poly(ADP-ribose) synthetase. Polymeric chains of poly(ADP-ribose) are covalently attached to DNA topoisomerase I. These observations with highly purified enzymes suggest that poly(ADP-ribosylation) may be a cellular mechanism for modulating DNA topoisomerase I activity in response to the state of DNA in the nucleus.

Although extensive poly(ADP-ribosylation) of the $M_r = 100,000$ DNA topoisomerase I from calf thymus resulted in >90% enzyme inhibition, exogenous poly(ADP-ribose) does not, by itself, inhibit topoisomerase activity. After modification, the apparent molecular weight of both the topoisomerase enzyme protein and of the topoisomerase enzyme activity was increased. *In vitro*, the extent of modification of DNA topoisomerase I could be controlled either by changing the ratio of topoisomerase to the synthetase or by varying the reaction time. More than 40 residues of ADP ribose per topoisomerase molecule could be added by the synthetase. Analysis of a poly(ADP-ribosylated) topoisomerase preparation that was about 50% inhibited revealed an average polymer chain length of 7.4, with 1–2 chains per enzyme molecule.

The nuclei of eukaryotic cells contain an enzyme, poly(ADP-ribose) synthetase, which uses NAD as a substrate and catalyzes the formation of a nucleic acid-like polymer, poly(ADP-ribose) (1, 2). Enzyme activity is dependent on the presence of double-stranded DNA (3, 4); DNA with nicks or linear DNA is stimulatory, but covalently closed circular DNA is not (5). Poly(ADP-ribose) is found covalently attached to a variety of acceptor proteins which, in somatic cells, include the synthetase itself (6, 7), histone H1 (8–11), the high mobility group proteins (11), and a Ca^{2+} , Mg^{2+} -dependent endonuclease (12).

Although the biological role for poly(ADP-ribose) has not been fully established, it has been implicated in an array of cellular events, all of which may involve changes in the structure of chromatin. Thus, a variety of experimental evidence has led to suggestions that the processes of DNA repair (13, 14), gene expression, cell differentiation and transformation (1, 2), and sister chromatid exchange (15–19) may involve the metabolism of poly(ADP-ribose). The biochemical mechanism for these postulated roles has not been elucidated.

DNA topoisomerases catalyze the concerted breakage and rejoining of the phosphodiester bonds of DNA and are one

class of enzyme that have the capacity to modify chromatin structure (20, 21). The nuclei of eukaryotic cells contain high levels of a type I DNA topoisomerase activity which can relax both positive and negative supercoils (in contrast to the first DNA topoisomerase isolated from *Escherichia coli* which only relaxes negatively supercoiled DNA (22)). This activity, first described from mouse embryos (23) and subsequently purified from a variety of eukaryotic cells (20, 21, 24–29), appears to account for the majority of the DNA topoisomerase activity in cell extracts.

Recently, we discovered that a DNA topoisomerase activity which co-purified with poly(ADP-ribose) synthetase from calf thymus was an acceptor for poly(ADP-ribose) (30). The modification resulted in an inhibition of the topoisomerase enzymatic activity. This discovery raised the possibility that some of the biological effects of poly(ADP-ribosylation) might be effected by modulating the activity of DNA topoisomerases. Preliminary studies indicated, however, that the topoisomerase activity which co-purified with the poly(ADP-ribose) synthetase was less than 0.1% of the total topoisomerase activity in extracts of thymus. Moreover, the major topoisomerase activity of calf thymus extracts was separated from poly(ADP-ribose) synthetase in the first column chromatographic step in the synthetase purification procedure (DNA agarose). Thus we were uncertain about the relationship between the co-purifying topoisomerase and the major calf thymus DNA topoisomerase I activity, and we felt it important to establish whether or not the major type I DNA topoisomerase activity in eukaryotic cells is a target for poly(ADP-ribosylation). In this report, we demonstrate that purified topoisomerase I from calf thymus is efficiently poly(ADP-ribosylated) and provide an initial biochemical characterization of the modification process.

EXPERIMENTAL PROCEDURES

Materials

Plasmid pBR322 was isolated from 6 liters of an *E. coli* strain C600 grown on L broth by a modification of the procedure of Birnboim and Doly (31, 32).¹ A yield of 2.5–6 mg of DNA was routinely obtained. Phosphodiesterase I (EC 3.1.4.1, from *Crotalus adamanteus* venom) was obtained from Sigma and purified by the method of Sulkowski and Laskowski (33). The preparation was made 50% in glycerol and contained 0.4 unit/ml. DNA agarose was prepared by the method of Schaller *et al.* (34) and contained 2 mg of DNA per ml of gel. Calf thymus (individually frozen organs) was obtained from Pel-Freez Biologicals, Rogers, AR, CsCl from Gallard-Schlesinger Corp., hydroxylapatite (Bio-Gel HTP) from Bio-Rad, ammonium sulfate from Schwarz/Mann, and [^{32}P]NAD (23 Ci/mmol) from New England Nuclear. All other biochemicals were obtained from Sigma.

General Methods

Assay procedures for DNA topoisomerase and poly(ADP-ribose) synthetase were described previously (30). Protein determinations were carried out by the method of Lowry *et al.* (35), using bovine

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¹ A. Laughon, personal communication.

serum albumin as a standard. Electrophoresis was carried out on polyacrylamide gels containing 0.1% SDS,² as described previously (36). In experiments in which poly(ADP-ribosylated) proteins were analyzed, the gel and sample buffers contained 6 M urea (37) and all buffers were pH = 7.0. Samples containing poly(ADP-ribosylated) proteins were stood at 22 °C for 20 min prior to electrophoresis; otherwise, samples were heated at 92 °C for 2 min before electrophoresis. To avoid cleavage of ADP-ribose-protein bonds during electrophoresis, the pH of electrode compartments was monitored and kept close to 7.0. Gels were stained with Coomassie brilliant blue G250. For autoradiographic analysis, the stained gels were dried (36).

Analysis of Native Molecular Weights of Poly(ADP-ribosylated) Proteins

Reaction mixtures (0.1 ml) contained 10 mM Tris-Cl, pH 8, 1 mM DTT, 10 mM magnesium chloride, 10 µg/ml of DNA, and poly(ADP-ribose) synthetase (7.5 µg). Control reactions also contained the 100,000-dalton DNA topoisomerase (5.6 µg) and were incubated at 23 °C for 20 min. Experimental reactions contained the DNA topoisomerase (11.2 µg) and in addition 2 mM [³²P]NAD (specific activity 21,000 cpm/nmol) and were incubated at 23 °C for 20 or 90 min. The incubation was terminated by the addition of nicotinamide (10 mM final concentration), and a mixture of proteins used for molecular weight markers (1.5 mg each of bovine IgG, bovine serum albumin, ovalbumin, and cytochrome c). The sample was applied to a Sephadex G-150 column (1.44 × 167 cm) which had been equilibrated against 50 mM Tris-Cl, pH 7.2, 0.35 M sodium chloride, 10% glycerol, and fractions (4.05 ml) were collected. Bovine serum albumin (400 µg) was added to each fraction to be assayed for enzymatic activity. The fractions were dialyzed for 3 h at 23 °C against 25 mM sodium bicarbonate, pH 10, 0.2 M NaCl chloride, 10% glycerol, and 2 mM DTT. This dialysis treatment strips off poly(ADP-ribose) and reactivates the enzyme, permitting assay (30, 36).

Extraction of Poly(ADP-ribose) from SDS-Urea Polyacrylamide Gels

Individual lanes were cut from the polyacrylamide slab gel (13 × 10.3 × 0.1 cm) immediately after the electrophoresis of the poly(ADP-ribosylated) proteins. The strips of gel were soaked in 10% isopropanol, 10% acetic acid, sliced into 2.5-mm segments, and the slices incubated in 1 M NH₄OH (pH = 11) containing 0.2 mg/ml of proteinase K at 37 °C for 24 h. This procedure extracted approximately 80% of the [³²P]poly(ADP-ribose) from the gel. The extracts (800 µl/slice) which contained the material of interest were pooled, and the resulting solutions were evaporated to dryness. The residues were dissolved in 200 µl of 5 mM Tris-Cl, pH 8.0. This solution was extracted with phenol (saturated with 0.1 M Tris-Cl, pH 8.0) and the aqueous phase extracted with water-saturated ether. The recovery of radioactivity for the phenol extraction step was approximately 70%. The analysis of poly(ADP-ribose) chain size was carried out on this fraction (200 µl).

Determination of Poly(ADP-ribose) Chain Size

Samples (100 µl) were made 1 mM in 5'-AMP and 5 mM in MgCl₂ and digested with 800 microunits of snake venom phosphodiesterase at 37 °C for 1 h. Paper chromatographic analysis using the isobutyric acid-30% NH₄OH-H₂O (66:1:33, v/v) solvent system (38) was carried out as described previously (39).

Enzyme Purifications

Extraction Buffer—This was a mixture of the following components: Tris base (50 mM), NaCl (300 mM), Na bisulfite (50 mM), glycerol (10%, v/v), β-mercaptoethanol (10 mM), adjusted to pH 8.0 with NaOH.

Dialysis Buffers a and b—This was extraction buffer diluted 1.5-fold (a) or 3-fold (b) with 10% glycerol, 10 mM β-mercaptoethanol.

Buffer A—This consisted of 50 mM Tris-Cl, pH 8.0, 2 mM DTT, 10% (v/v) glycerol.

Purification of DNA Topoisomerase from Calf Thymus

All operations were carried out at 2 °C. All centrifugations were carried out at 6800 × g unless otherwise indicated. As reported previously (36), extracts of thymus obtained from certain animals

had very unstable poly(ADP-ribose) synthetase activity. Since poly(ADP-ribose) synthetase and DNA topoisomerase could be isolated from the same batch of thymuses, organs were selected which yielded stable extracts of the synthetase. Portions (5 g) of individual frozen thymuses were homogenized in extraction buffer, centrifuged, and the supernatant fractions assayed the next day for poly(ADP-ribose) synthetase activity. Both enzymes were isolated from organs which yielded extracts with greater than 30 units of synthetase/g of tissue. (One unit of enzyme activity catalyzed the conversion of 1 nmol of NAD into an acid-insoluble form per min.) It is possible that this selection of organs may be important in determining the molecular weight of the DNA topoisomerase activity obtained.

I. Crude Extract—1.15 kg of calf thymus (13 organs) was minced with a meat grinder and homogenized in 5 volumes of extraction buffer. Extracts were made from 1 to 2 thymuses at a time, and each extract kept separate for this and the following purification step. Each homogenate was centrifuged for 10 min. To the supernatant fraction was added 3.75% protamine sulfate (20 ml/100 g of tissue) and the resulting suspension centrifuged for 10 min (Fraction I).

II. Ammonium Sulfate Fraction—To Fraction I was added solid ammonium sulfate to 40% saturation. After centrifugation for 20 min, additional ammonium sulfate was added to the supernatant fraction to give 80% saturation, and a second centrifugation was carried out. The second ammonium sulfate precipitate (the 40–80% fraction) was dissolved in dialysis buffer b and dialyzed extensively overnight. Insoluble material arising in the dialysis tube was removed by centrifugation for 20 min at 14,500 × g (Fraction II). Ammonium sulfate fractions were discarded which had less than a 40% recovery of poly(ADP-ribose) synthetase activity relative to Fraction I. The steps described above were carried out in 24 h.

III. DNA Agarose Chromatography—The ammonium sulfate fraction (Fraction II, 432 ml) was applied to a DNA agarose column (21.5 × 4.2 cm) at a rate of 1.4 ml/min. The column had previously been equilibrated with dialysis buffer b. After application of the sample, the column was washed with 500 ml of dialysis buffer b, followed by buffer A containing a linear gradient of 0.1–0.45 M NaCl (total volume, 1 liter). The topoisomerase eluted between 0.25–0.35 M NaCl, and active fractions were pooled (Fraction III).

IV. Hydroxylapatite Column Chromatography—The DNA agarose fraction (240 ml) was applied to hydroxylapatite column (9.5 × 2.5 cm) equilibrated with buffer A plus 0.2 M sodium chloride. After application, the column was washed with 100 ml of buffer A plus 0.2 M sodium chloride, followed by 120 ml of buffer A plus 0.2 M sodium chloride, 50 mM potassium sulfate, pH 8.0, followed by 50 mM Tris chloride, pH 7.0, 0.2 M sodium chloride, 10% glycerol, 2 mM DTT, and a final wash (30 ml) of 2.5 mM potassium phosphate, pH 7.2, 2 M KCl, 10% glycerol, 2 mM DTT. The enzyme was eluted using a linear gradient of potassium phosphate, pH 7.2 (from 2.5 to 300 mM) containing 2 M KCl, 10% glycerol, 2 mM DTT (total volume, 400 ml). The topoisomerase activity was eluted between 50 to 100 mM potassium phosphate. The active fractions were pooled (Fraction IV).

V. Sephadex G-150 Chromatography—The hydroxylapatite fraction (54 ml) was dialyzed against buffer A containing 0.2 M sodium chloride and saturated with ammonium sulfate for 12 h at 2 °C. The turbid suspension was centrifuged and the pellet dissolved in buffer A plus 0.2 M sodium chloride (final volume 3 ml). This sample was applied to a Sephadex G-150 column (165.5 × 2.67 cm) equilibrated against buffer A plus 0.2 M sodium chloride. Fractions were collected (3.55 ml) and active fractions containing topoisomerase activity were pooled and concentrated by precipitation with ammonium sulfate, as described above. The concentrated fractions were dialyzed against 30 mM potassium phosphate, pH 7.2, 2 mM DTT, 0.1 mM EDTA, 50% (v/v) glycerol (25). Final volumes for individual pools were approximately 0.35 ml; these were stored for several months at –20 °C with no loss of enzyme activity (Fraction V).

Purification of Poly(ADP-ribose) Synthetase from Calf Thymus

The synthetase preparation used in this study was purified from 1.19 kg of tissue using a modification of the procedure of Ferro and Olivera (36). The DNA topoisomerase purification scheme described above uses essentially the same steps as the procedure for the purification of the synthetase. The preparation of Fraction I (crude extract, 5.5 liters) and Fraction II (ammonium sulfate fraction) was done as described previously (36) except that the steps were carried out in the same day and extracts were made from 1 to 2 thymuses at a time and kept separate until the DNA agarose step. The individual ammonium sulfate pellets were dissolved in dialysis buffer a and dialyzed

² The abbreviations used are: SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

overnight. This dialysis step replaced the desalting step on Sephadex G-25 (36) since it was easier in this way to simultaneously desalt several samples. Insoluble material resulting from the dialysis step was removed by centrifugation at $14,500 \times g$ for 20 min. The dialyzed fraction (465 ml) was applied to a DNA agarose column as described above for the preparation of DNA topoisomerase, except that the column was equilibrated with buffer A containing 0.2 M NaCl. After application of the sample, the column was washed with 500 ml of buffer A containing 0.2 M NaCl, followed by buffer A containing a linear gradient of 0.2–1.5 M NaCl (total volume, 1 liter). Poly(ADP-ribose) synthetase eluted at approximately 1 M NaCl; active fractions were pooled (362 ml). The DNA agarose column chromatographic step was changed somewhat from the previous report (36). The modifications are an increase in both the total volume of the column (25 ml of gel/100 g of tissue) and the DNA concentration of the gel (2 mg of DNA/ml of gel). The DNA agarose column thus had a higher capacity to bind the poly(ADP-ribose) synthetase activity in the ammonium sulfate fraction, and little enzyme activity was lost in the pass-through fraction. The DNA agarose fraction was chromatographed on hydroxylapatite, exactly as described above, for the preparation of DNA topoisomerase, and the fractions containing enzyme activity were pooled. This procedure was changed from the hydroxylapatite step reported earlier (36), in order to remove residual nucleic acid (30).

The hydroxylapatite fraction was concentrated, chromatographed on Sephadex G-150, and then reconcentrated, following the procedure described above for the preparation of DNA topoisomerase (Fraction V). These steps were essentially the same as those reported earlier (36) for the purification of poly(ADP-ribose) synthetase, except that the final concentrated enzyme preparation was dialyzed against buffer A containing 0.2 M NaCl, 50% (v/v) glycerol. The enzyme preparation had a single polypeptide component when electrophoresed on SDS-polyacrylamide gels and had a specific activity of 800 units/mg of protein. This was slightly higher than the specific activity of the best fraction of synthetase prepared by the original method (36). Moreover, the activity of the enzyme prepared by the modified method was almost totally dependent on the addition of DNA to the assay mixture. The preparation was stored for several months at -20°C with no loss of activity.

RESULTS

Purification of DNA Topoisomerase—DNA topoisomerase I was purified from calf thymus; a summary of the purification procedure is shown in Table I.

Fig. 1 shows a profile of enzymatic activity upon gel filtration on Sephadex G-150, the last step in the purification procedure. A broad peak of DNA topoisomerase activity was detected, suggesting that several molecular weight species might be present. Fractions from the Sephadex column were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1b). The leading edge of the peak of enzymatic activity contained predominantly one polypeptide component with a molecular weight of 100,000. However, enzymatic activity was also found in fractions from the column that had as major components proteins of molecular weight 85,000, 77,000, and 69,000 on

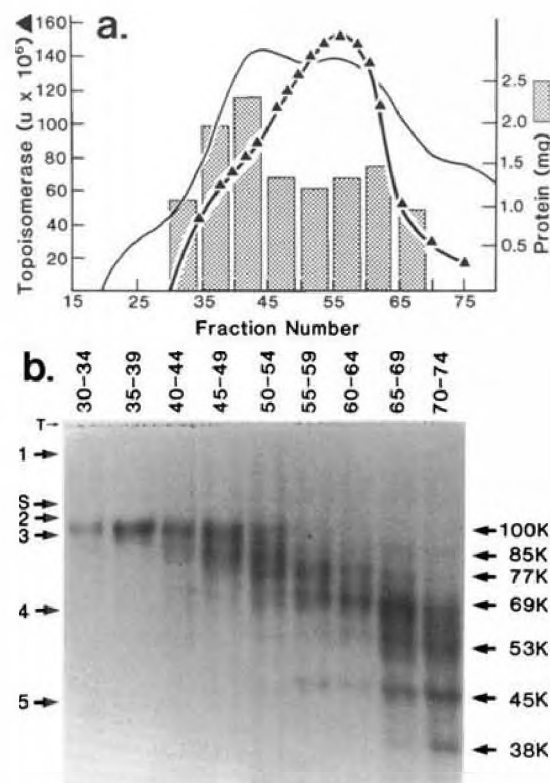


FIG. 1. Chromatography on Sephadex G-150. *a*, Sephadex G-150 column chromatography, the last step in the purification of the DNA topoisomerase, was carried out as described under "Experimental Procedures." Enzyme units and protein concentrations refer to the total amounts per fraction. Fluorescence units are arbitrary (shown by the thin line). *b*, the various fractions from the Sephadex G-150 column were analyzed by electrophoresis on 7% polyacrylamide gels containing SDS (no urea; see under "Experimental Procedures."). The pooled fractions of the column in *a*, which were analyzed in each lane, are indicated at the top of the gel. Arrows on left refer to the positions of marker proteins run on the same gel. The proteins, with their denatured molecular weights, were: 1, rabbit muscle myosin (heavy chain) 200,000; 2, *E. coli* β -galactosidase, 116,000; 3, rabbit muscle phosphorylase α , 94,000; 4, bovine serum albumin, 68,000; 5, chicken egg albumin, 43,000; *T* indicates the top of separating gel. The arrows on the right indicate the major polypeptide components with their estimated molecular weights based on a relationship between the logarithm of the molecular weight of the marker proteins versus mobility. The 100,000 molecular weight component comprised approximately 40% of the protein in fractions 30–69. 100K, for example, $M_r = 100,000$, etc.

TABLE I
Purification of DNA topoisomerase

Fraction	Total protein mg	Total activity units $\times 10^6$	Specific activity units $\times 10^6$ /mg
I. Crude extract	16,360	13.2 ^b	0.81
II. Ammonium sulfate	7,470	21.0	2.8
III. DNA agarose	230	10.2	44.3
IV. Hydroxylapatite	55.0	7.0	127
V. Sephadex G-150 ^c	11.8	3.5	296
Va. Sephadex G-150 ^d	2.0	0.36	196

^a One unit of DNA topoisomerase activity relaxed 50% of the plasmid DNA under the conditions of the assay (30).

^b The topoisomerase activity in the supernatant fraction from the tissue homogenate was recovered nearly quantitatively in Fraction I.

^c Totals for fractions 30–69.

^d Fractions 35–39. The specific activity shown is the average for several determinations.

SDS-gel electrophoresis. As shown in Fig. 2, when the leading edge of the Sephadex G-150 fraction was pooled and rechromatographed on Sephadex G-150, a symmetrical peak of protein fluorescence was eluted from the column with a coincident peak of DNA topoisomerase activity. The specific activity of the 100,000-dalton DNA topoisomerase is approximately 2.0×10^6 units/mg, which corresponds to that reported for the purified enzyme from HeLa cells with the same molecular weight (25). It should be noted that fractions which were more retarded on the Sephadex column had apparent specific activities higher than that of the 100,000 dalton component (i.e. $4\text{--}5 \times 10^6$ units/mg). The higher specific activities of the lower M_r topoisomerases may translate into equivalent molar specific activities; in addition, a more processive enzyme might appear less active given our present assay conditions.

Previously published purification procedures for Type I topoisomerase from calf thymus yielded preparations with

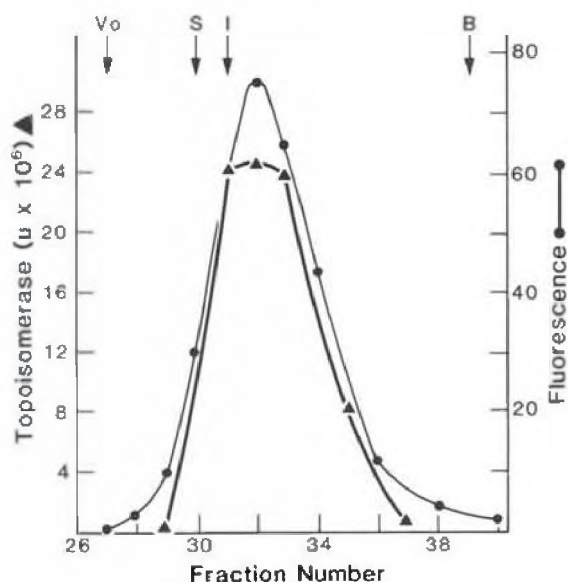


FIG. 2. Rechromatography of the 100,000-dalton DNA topoisomerase I on Sephadex G-150. An aliquot of the pool of fractions 35–39 shown in Fig. 1a was applied to a Sephadex G-150 column (167 × 1.4 cm) equilibrated against buffer A plus 0.2 M sodium chloride. Fractions (4.1 ml) were collected, fluorescence was measured in arbitrary units, and DNA topoisomerase activity was assayed as described under "Experimental Procedures." The arrows indicate the excluded volume (V_0) and the elution positions of: poly(ADP-ribose) synthetase (S), bovine IgG (I), and bovine serum albumin (B).

molecular weights from 32,000–75,000 daltons (40, 41). However, by minimizing proteolytic degradation, preparations of $M_r = 100,000$ DNA topoisomerase I from a mammalian cell line (25) and from chicken erythrocytes (29) have been obtained. For the HeLa cell topoisomerase I, it was shown that an enzymatically active $M_r = 67,000$ polypeptide was a proteolytic fragment of the $M_r = 100,000$ form (25). Although purification procedures for the $M_r = 100,000$ topoisomerase have not been published to date for calf thymus or any mammalian tissue, the work with HeLa topoisomerases makes it likely that the smaller molecular weight species of topoisomerase isolated are proteolytic products of the $M_r = 100,000$ form. In our procedure they co-purify through all but the last column.

Inhibition of DNA Topoisomerase Activity by Poly(ADP-ribosylation)—The 100,000 dalton DNA topoisomerase I was incubated with purified poly(ADP-ribose) synthetase. As shown in Table II, the DNA topoisomerase was found to be severely inhibited after incubation with the synthetase in the presence of NAD and DNA.

The observed inhibition required incubation of the topoisomerase and poly(ADP-ribose) synthetase together; when DNA topoisomerase was added after poly(ADP-ribose) synthetase was incubated with NAD, inhibition was not observed although substantial levels of poly(ADP-ribose) were synthesized and, therefore, present during the topoisomerase assay. These results indicate that the presence of poly(ADP-ribose) is not inhibitory and suggest that topoisomerase inhibition may be due to direct poly(ADP-ribosylation) of the topoisomerase protein.

Physical Evidence for Poly(ADP-ribosylation) of DNA Topoisomerase—The DNA topoisomerase was analyzed by polyacrylamide gel electrophoresis after poly(ADP-ribosylation) (Fig. 3). With increasing incubation time in a reaction mixture that contained poly(ADP-ribose) synthetase (2 $\mu\text{g}/\text{ml}$) and a 25-fold molar excess of DNA topoisomerase (40 $\mu\text{g}/\text{ml}$), the

TABLE II
Inhibition of the $M_r = 100,000$ topoisomerase by poly(ADP-ribosylation)

Reaction mixtures (200 μl) contained 10 mM Tris-Cl, pH 8.0, 1 mM DTT, 10 mM MgCl_2 , 10 $\mu\text{g}/\text{ml}$ of DNA, 0.2 mM NAD, [^{32}P]NAD (final NAD specific activity = 27,000 cpm/nmol), and aliquots of the enzyme preparation, as shown. NAD incorporation was assayed during the course of the reaction as described earlier (36). After a 20-min incubation at 23 °C, an aliquot (10 μl) was quenched by the addition of nicotinamide (10 mM final), and synthetase (80 ng) or topoisomerase (145 ng) were added. Topoisomerase activity was then assayed (see under "Experimental Procedures"). ND, not determined.

Reaction number	Topoisomerase ^a $\mu\text{g}/\text{ml}$	Poly(ADP-ribose) synthetase $\mu\text{g}/\text{ml}$	Incubation time min	NAD incorporated nmol/ml	Added with nicotinamide quench	Topoisomerase activity units/ml, $\times 10^{-6}$
1		8	5	13.5	Topoisomerase	1.6
			20	27.5		
2	14.5		20	<2.5	Synthetase	2.3
3	14.5	8	5	16.5		ND
			20	25.0		0.17

^a Fraction Va, Table I.

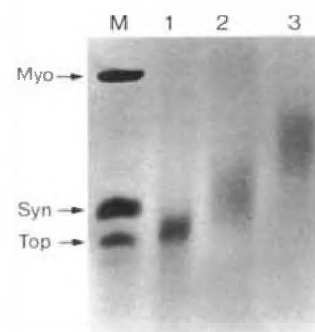


FIG. 3. Poly(ADP-ribosylation) of the $M_r = 100,000$ DNA topoisomerase: kinetic analysis. The reaction mixture (250 μl) contained 10 mM Tris, pH 8.0, 1 mM DTT, 10 mM MgCl_2 , 10 $\mu\text{g}/\text{ml}$ of DNA, 0.2 mM [^{32}P]NAD (final specific activity, 2.8×10^6 cpm/nmol), 2 $\mu\text{g}/\text{ml}$ of poly(ADP-ribose) synthetase, and 40 $\mu\text{g}/\text{ml}$ of DNA topoisomerase. The sample was incubated at 22 °C for various times (0.75, 4, 20 min), and 80- μl aliquots were withdrawn and added to 300 μl of 20% trichloroacetic acid at 0 °C. The suspension was centrifuged and the pellet washed twice with 10% trichloroacetic acid and once with diethyl ether. Electrophoresis was carried out on a 5% polyacrylamide slab gel containing SDS and urea (see under "Experimental Procedures"). Myo, rabbit muscle myosin (heavy chain, $M_r = 200,000$); Syn, poly(ADP-ribose) synthetase, $M_r = 120,000$; Top, DNA topoisomerase I, $M_r = 100,000$. Lane M was loaded with marker proteins; lanes 1, 2, and 3 were loaded with the reaction mixture which had been incubated for 0.75, 4, and 20 min, respectively. After the 20-min reaction, 29 nmol of NAD had been incorporated into an acid-insoluble form per ml of reaction, and the DNA topoisomerase was inhibited 3-fold relative to the control (the unmodified topoisomerase).

migration of the DNA topoisomerase protein (stained with Coomassie blue) decreased in the gel. After a 20-min reaction, the protein band moved to a position midway between poly(ADP-ribose) synthetase ($M_r = 120,000$) and myosin heavy chain ($M_r = 200,000$), at an apparent molecular weight of approximately 175,000. The concentration of poly(ADP-ribose) synthetase was below the level of detection by Coomassie staining. As the reaction proceeded, all of the DNA topoisomerase molecules had a retarded mobility in the gel. The "apparent molecular weights" of modified DNA topoisomerase obtained from gel electrophoresis do not represent the true molecular weights of the poly(ADP-ribosylated) protein. However, there is linear relationship between retardation of

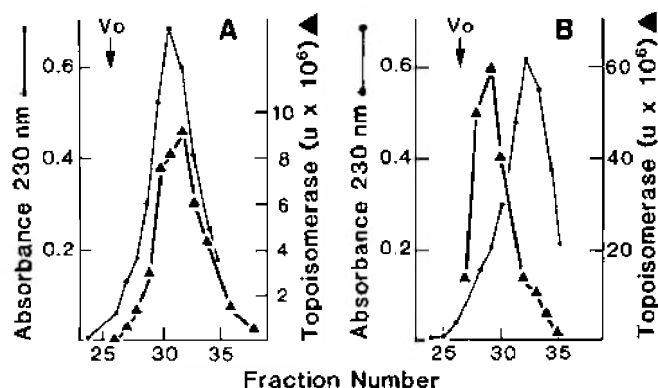


FIG. 4. Increase in molecular size of the 100,000-dalton DNA topoisomerase activity after poly(ADP-ribosylation). A sample of DNA topoisomerase which had not been poly(ADP-ribosylated) (A), and one which had been extensively poly(ADP-ribosylated) (B), were prepared as described under "Experimental Procedures." The experimental sample shown on the right was incubated for 90 min with poly(ADP-ribose) synthetase; 25 nmol of NAD were incorporated into acid-insoluble material. The samples were applied to a Sephadex G-150 column with a mixture of proteins used for molecular weight markers (IgG, bovine serum albumin, ovalbumin, and cytochrome c). The absorbance profile corresponds to the IgG peak; elution positions for the other marker proteins are not shown. The elution position of blue dextran is indicated by the arrow (labeled V_0). Fractions from the column were assayed for DNA topoisomerase activity (see under "Experimental Procedures"). The apparent molecular weight of unmodified DNA topoisomerase is 190,000 and that of the poly(ADP-ribosylated) topoisomerase is greater than 300,000. The topoisomerase activity profile shown in B was coincident with a peak of [32 P]poly(ADP-ribose) which had been formed during the poly(ADP-ribosylation) reaction described under "Experimental Procedures." (data not shown).

mobility and the logarithm of the degree of modification.³

Although the results above strongly suggest that the DNA topoisomerase is poly(ADP-ribosylated), it is necessary to demonstrate not only that the major protein band in the preparation is shifted after poly(ADP-ribosylation), but that the DNA topoisomerase enzymatic activity itself becomes associated with a protein of higher molecular weight. We used gel filtration as a criterion. The unmodified 100,000-dalton DNA topoisomerase, when co-chromatographed on Sephadex G-150 with IgG, eluted slightly behind the IgG marker (apparent molecular weights 205,000 and 190,000, respectively) (Fig. 4). After extensive poly(ADP-ribosylation) in a reaction mixture that contained a 1.8-fold molar excess of topoisomerase (11.2 μ g/ml of topoisomerase and 7.5 μ g/ml of poly(ADP-ribose) synthetase), the topoisomerase activity was again analyzed by chromatography on Sephadex G-150. Since poly(ADP-ribosylation) inhibits the topoisomerase, fractions from the Sephadex column were dialyzed at pH 10 to break the protein-poly(ADP-ribose) linkages, thereby reactivating the topoisomerase (30, 36); each fraction was then assayed for topoisomerase activity. After 20 min, the topoisomerase activity was shifted to a position slightly ahead of the IgG marker (results not shown); after 90 min, topoisomerase activity was found to elute well ahead of the IgG marker with an apparent molecular weight greater than 300,000 (Fig. 4). These experiments, in conjunction with the analysis using SDS-urea gel electrophoresis show that poly(ADP-ribosylation) significantly changes the apparent molecular size of the 100,000-dalton DNA topoisomerase of calf thymus.

Relative ADP-ribosylation of Topoisomerase and Synthetase—An initial step in characterizing the modification of

DNA topoisomerase I is to determine the extent of ADP-ribosylation of topoisomerase under a variety of reaction conditions. This is not a straightforward determination since poly(ADP-ribose) synthetase not only modifies the topoisomerase but carries out auto-poly(ADP-ribosylation). At the end of the reaction, ADP-ribose moieties are attached to both enzymes and, therefore, the proportion attached to topoisomerase must be independently determined. We have quantified ADP-ribose moieties attached to the topoisomerase and to the synthetase in reaction mixtures in which the ratio between the two enzymes has been systematically varied.

The poly(ADP-ribosylated) topoisomerase was separated from poly(ADP-ribosylated) synthetase using SDS-urea polyacrylamide gel electrophoresis. The poly(ADP-ribose) associated with the synthetase and the topoisomerase was distinguished by autoradiography and quantitated by measuring the radioactivity in gel slices (Fig. 5a). As the topoisomerase concentration was increased in a poly(ADP-ribosylation) reaction mixture containing a fixed concentration of synthetase (2 μ g/ml), the rate of total poly(ADP-ribose) formation increased (Fig. 5b, solid line).

Increasing the concentration of topoisomerase led to an increased incorporation of poly(ADP-ribose) into the topoisomerase fraction (Fig. 5b), but the rate of automodification decreased slightly. However, the number of ADP-ribose residues per topoisomerase enzyme molecule decreased with increasing topoisomerase in the reaction mixture. Thus, at concentrations of 20, 40, and 80 μ g/ml, the ADP-ribose residues per topoisomerase molecule decreased (to 41, 36, and 20, respectively). This variation in the extent of modification of the topoisomerase was reflected in the mobility of the poly(ADP-ribosylated) topoisomerase in the gel (Fig. 5a, lanes 3–5). At topoisomerase concentrations below 20 μ g/ml (holding the synthetase concentration constant at 2 μ g/ml), the mobility of the modified topoisomerase was so low that it was not fully resolved from the automodified synthetase (Fig. 5a, lane 2), and estimates of the extent of topoisomerase modification in this concentration range were not possible. These data indicate, however, that the extent of topoisomerase modification *in vitro* can be extensive (greater than 40 ADP-ribose residues/topoisomerase molecule) under the appropriate reaction conditions. Indeed, the extent of inhibition of DNA topoisomerase activity by poly(ADP-ribosylation) might be used to indirectly assess the extent of modification. Inhibition increases as the topoisomerase concentration is lowered in the standard poly(ADP-ribosylation) reaction. At a topoisomerase/synthetase molar ratio = 12.5 (20 μ g/ml of topoisomerase, 2 μ g/ml of synthetase) the topoisomerase activity was inhibited 3-fold when assayed after a 20-min poly(ADP-ribosylation) reaction. When the ratio was reduced to 0.78 (1.25 μ g/ml of topoisomerase), a 9-fold inhibition was observed (data not shown).

Analysis of ADP-Ribose Bound to DNA Topoisomerase—A more detailed analysis of ADP-ribose moieties attached to DNA topoisomerase I was carried out under conditions of relatively limited modification of the topoisomerase. The aim of this study was to obtain definitive chemical evidence for the presence of polymeric chains and to evaluate the extent of mono(ADP-ribosylation) (for histone H1, the best studied exogenous acceptor, both poly(ADP-ribose) and mono(ADP-ribose) have been reported (8–11, 42, 43)).

We modified the protocol described in Fig. 5 to achieve a maximum separation of the two modified enzymes using the same gel electrophoresis system. The basic strategy was to increase automodification of the synthetase and decrease ADP-ribosylation of the topoisomerase. The poly(ADP-ri-

³ A. Ferro, unpublished results.

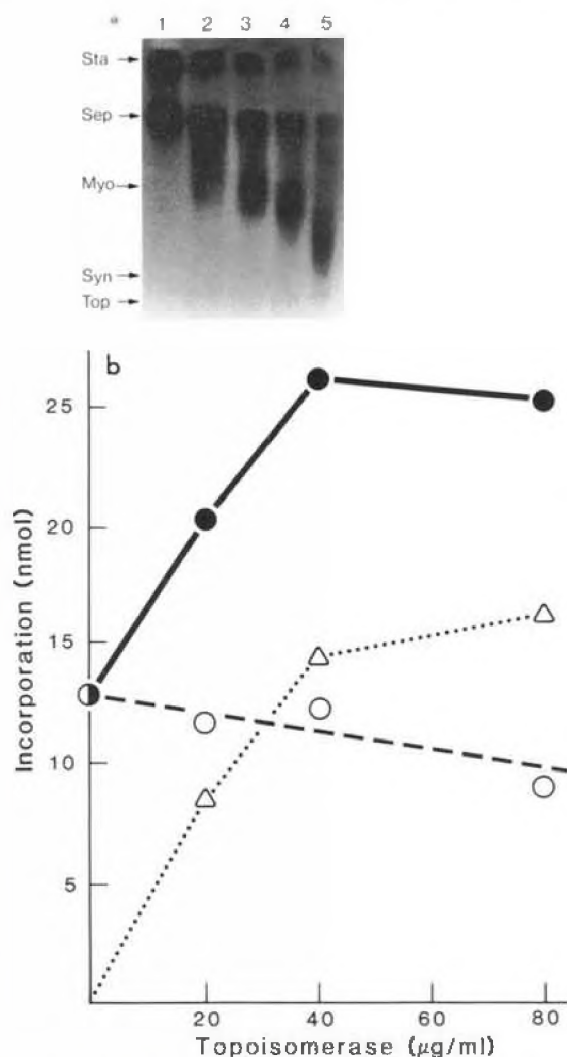


FIG. 5. Poly(ADP-ribosylation) of the $M_r = 100,000$ DNA topoisomerase: effect of the topoisomerase concentration. The reaction conditions were the same as described in the legend to Fig. 3, except that the topoisomerase concentration was varied from 0 to 80 $\mu\text{g/ml}$ of reaction, and the specific activity for NAD was 0.4×10^6 cpm/nmol. At 20 min, the reaction was terminated with 20% trichloroacetic acid, and aliquots were analyzed for NAD incorporation (36) or processed and electrophoresed as described in the legend to Fig. 3. The gel was stained with Coomassie blue, dried, and analyzed autoradiographically (a). Sta, top of stacking gel (3.3% polyacrylamide); Sep, top of separating gel (5.0% polyacrylamide); Myo, Syn, Top, see legend to Fig. 3. Lanes 1–5, 0, 5, 20, 40, and 80 $\mu\text{g/ml}$ of topoisomerase, respectively. Individual lanes were cut from the dried slab gel, the lanes sliced into 2.5-mm segments, and the radioactivity measured in the slices (36). Thus the [^{32}P]poly(ADP-ribose) associated with the synthetase and the topoisomerase could be separated and quantitated (b). Incorporation is expressed as nanomoles of ADP-ribose residues/ml of reaction. ●, total incorporation; ○, incorporation into the synthetase fraction; △, incorporation into the DNA topoisomerase fraction.

bosc) synthetase was preincubated (1 min) in the reaction mixture before a high level (80 $\mu\text{g/ml}$) of DNA topoisomerase was added. Since automodification during the preincubation caused an inhibition of the synthetase (36), the DNA topoisomerase became relatively less modified.

An analysis of the separation between the two modified enzymes is shown in Fig. 6. The extent of ADP-ribosylation of DNA topoisomerase was decreased (from 20 ADP-ribose moieties per enzyme molecule in the experiment in Fig. 5 to 12 ADP-ribose moieties per topoisomerase molecule). The

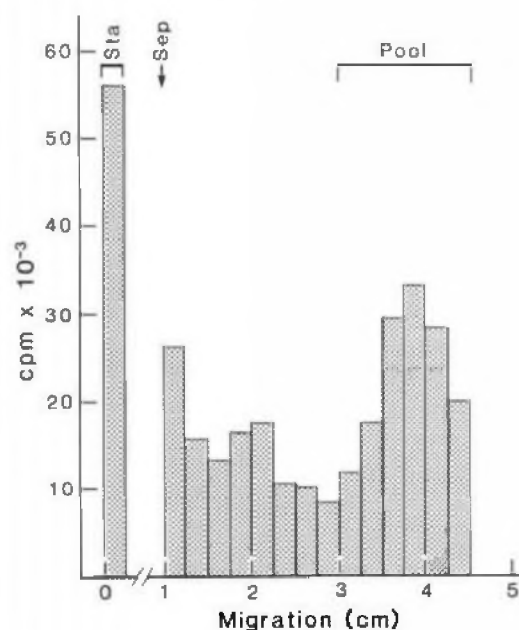


FIG. 6. Isolation of the poly(ADP-ribose) chains which modify DNA topoisomerase. DNA topoisomerase was poly(ADP-ribosylated) under the reaction conditions described in the legend to Fig. 3 except that the topoisomerase concentration was increased to 80 $\mu\text{g/ml}$, and, in addition, the topoisomerase was added to the reaction mixture 1 min after the poly(ADP-ribose) synthetase. The reaction mixtures were incubated for 20 min and processed for gel electrophoresis as described in the legend to Fig. 3. Immediately after electrophoresis, the lane was cut from the slab gel. The lane was then soaked, sliced, and the [^{32}P]poly(ADP-ribose) extracted, as described under "Experimental Procedures." Total [^{32}P]radioactivity in the various slices is shown in the figure. The [^{32}P]poly(ADP-ribose) that migrated from 3.0–4.5 cm in the gel was pooled. Sta, 1-cm stacking gel (one slice); Sep, the top of the separating gel, which was sliced into 2.5-mm segments (see under "Experimental Procedures").

radioactivity traveling with the DNA topoisomerase protein peak was eluted from the gel and released from the protein by treatment with protease and mild alkali. When the radioactivity in the ADP-ribose moieties was analyzed by paper chromatography, it was found at the origin of the chromatogram. There was essentially no detectable (<6%) mono- and di-ADP-ribose (Fig. 7A).

The polymeric chains were broken down using venom phosphodiesterase; the major products of digestion were phosphoribosyl-AMP and AMP. These data indicate that the average chain size under these reaction conditions was 7.4, and less than one in five chains was branched (radioactivity traveling at the R_f of the branch structure (44) was barely detectable above background). DNA topoisomerase so modified retained approximately 50% of its activity.

Thus, data show that even under these conditions of relatively limited modification, each enzyme molecule is poly(ADP-ribosylated). Under most reaction conditions, the poly(ADP-ribose) synthetase would catalyze more extensive *in vitro* modification of DNA topoisomerase leading to greater topoisomerase inhibition.

DISCUSSION

Poly(ADP-ribose) synthetase and DNA topoisomerase I are found ubiquitously, and at relatively high levels, in the nuclei of higher eukaryotes (1, 2, 20, 21, 24–29). The biological role of these enzymes is incompletely understood. In this work we have demonstrated that DNA topoisomerase I from calf thymus is a target for poly(ADP-ribosylation), resulting in at-

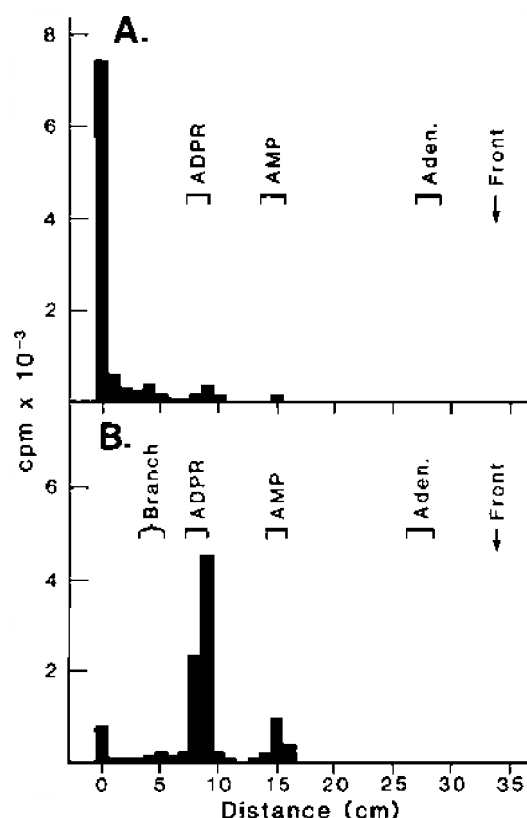


FIG. 7. Estimation of the chain size of poly(ADP-ribose) which modifies DNA topoisomerase. The poly(ADP-ribose) component of the modified DNA topoisomerase was isolated, as described in the legend to Fig. 6. The poly(ADP-ribose) was digested with snake venom phosphodiesterase and the digest chromatographed on paper, as described under "Experimental Procedures." In A, undigested poly(ADP-ribose) was chromatographed; B, the products of digestion were chromatographed. For this experiment, the chain size was calculated (44) to be 7.4. Brackets at top are the positions of authentic markers which were co-chromatographed with the sample. Aden., adenosine; Branch, the position on the chromatogram where 2'-[1''-ribosyl-2''-(1'''-ribosyl)]adenosine-5',5'',5'''-Tris(phosphate) would run (44); ADPR, ADP-ribose; Front, solvent front.

tachment of poly(ADP-ribose) chains to the topoisomerase and inactivation of the enzyme. Our results were obtained using the large molecular weight form (100,000) of calf thymus DNA topoisomerase I. The smaller molecular weight forms of DNA topoisomerase may be enzymatically active proteolytic fragments of the $M_r = 100,000$ form (25, 29). Poly(ADP-ribosylation) of these smaller forms was, therefore, not studied in detail, although it was observed that they were subject to inhibition by modification.

We have provided chemical evidence that the covalent modification of DNA topoisomerase by the synthetase involves poly(ADP-ribose) and that mono(ADP-ribosylation) is not significant. Thus, the (ADP-ribose)_x residues which migrated with the topoisomerase on SDS-urea gel electrophoresis were subjected to proteolysis and to alkaline conditions which split the (ADP-ribose)_x-protein linkage (37). The material released does not migrate from the origin on paper chromatography, while under these conditions mono- or di-(ADP-ribose) residues migrate significantly (see Fig. 7A). Furthermore, when the (ADP-ribose)_x residues attached to the topoisomerase protein are digested with venom phosphodiesterase, the predominant product is phosphoribosyl-AMP which could only have come from internal positions in a poly(ADP-ribose) chain. In the experiment shown in Fig. 7,

the average chain length of poly(ADP-ribose) attached to DNA topoisomerase was 7.4; essentially all topoisomerase molecules had at least one poly(ADP-ribose) chain attached. This degree of poly(ADP-ribosylation) lowers the activity of DNA topoisomerase I approximately 50%. When poly(ADP-ribosylation) reactions are carried out with lower ratios of topoisomerase to synthetase and with longer incubation times, a significantly greater extent of modification of the topoisomerase can be achieved (>40 ADP-ribose residues/enzyme) with a correspondingly greater inhibition of enzyme activity (<10% activity remaining). A detailed analysis of more highly poly(ADP-ribosylated) species of DNA topoisomerase I requires the development of methods which cleanly separate extensively poly(ADP-ribosylated) topoisomerase from extensively automodified poly(ADP-ribose) synthetase.

The modification of DNA topoisomerase I is presumably occurring at the DNA sites which activate poly(ADP-ribose) synthetase. Even with a 50-fold molar excess of DNA topoisomerase over poly(ADP-ribose) synthetase, every topoisomerase molecule becomes modified under the reaction conditions used. The extent of poly(ADP-ribosylation) of each topoisomerase molecule continues to increase with time; thus, the modification reaction is not highly processive under these reaction conditions. Furthermore, the marked inhibition of DNA topoisomerase I by poly(ADP-ribosylation) which is achieved even when both enzymes are present at the concentration of $<5 \times 10^{-6}$ M suggests either very high affinity between the two enzymes or high affinity for the same site on the activating DNA.

How does extensive poly(ADP-ribosylation) of DNA topoisomerase lead to the loss of enzymatic activity? For poly(ADP-ribose) synthetase, we previously postulated that the large increase in net negative charge upon poly(ADP-ribosylation) eventually causes repulsion between the activating DNA and the automodified synthetase (36). This concept of a "repulsion point" was sufficient to rationalize many of the details of the automodification reaction. A similar explanation for inactivation of modified DNA topoisomerase I seems reasonable. However, *in vivo* the topoisomerase inhibition would presumably be temporary. Since the intracellular half-life of poly(ADP-ribose) is a few minutes (45), poly(ADP-ribosylation) of DNA topoisomerase I may not persist unless the synthetase was maintained in an activated form.

Finally, these results raise the possibility that some of the phenomenology established for poly(ADP-ribosylation) might be explained by DNA topoisomerase I modification. For example, it is well established that inhibitors of poly(ADP-ribose) synthetase stimulate the rate of sister chromatid exchange (15-19). The discovery that DNA topoisomerase I is inhibited could provide a plausible enzymatic mechanism for this observation. Eukaryotic DNA topoisomerase I catalyzes a strand exchange *in vitro* (46); the covalent protein DNA intermediate in the topoisomerase reaction is attacked by a DNA strand with a 5'-hydroxyl terminus, linking the exogenous DNA through a phosphodiester bond (results consistent with homologous strand exchange have also been obtained using yeast DNA topoisomerase I (28)). Since poly(ADP-ribose) synthetase is stimulated when such 5'-OH-terminated DNA strand breaks are present (5), the enzyme would presumably poly(ADP-ribosylate) and inhibit DNA topoisomerase I *in vivo*. However, if synthetase inhibitors are added, DNA topoisomerase I would not be modified. Thus, the increased frequency of sister chromatid exchange could arise from the failure to inhibit DNA topoisomerase I in the vicinity of DNA strand breaks.

The function of poly(ADP-ribose) synthetase in chromatin

metabolism has been the subject of considerable speculation (1, 2). The modulation of DNA topoisomerase I activity by poly(ADP-ribosylation) provides one possible mechanism, which can be systematically investigated, through which these yet undefined biological functions of the synthetase can be achieved.

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